Addendum

Invention Title

Nucleotide Sequences Which Code for the eno Gene

APPLICATION UNDER UNITED STATES PATENT LAWS

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Invention: NUCLEOTIDE SEQUENCES WHICH CODE FOR THE ENO GENE

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SPECIFICATION

New Nucleotide Sequences which Code for the Eno Gene

Field of the Invention

The present invention provides nucleotide sequences coding for a bacterial enolase enzyme. It encompasses processes for the fermentative preparation of amino acids using coryneform bacteria in which the eno gene is amplified.

Background of the Invention

L-amino acids, especially L-lysine, are used in the feeding of animals, in human medicine and in the pharmaceutical industry. They are typically produced by fermenting strains of coryneform bacteria, especially Corynebacterium glutamicum. Because of the great importance of amino acids, work is continually being done to improve production processes. Improvements may concern measures relating to the fermentation process (*e.g.*, relating to stirring and oxygen supply) or the composition of the nutrient medium, (*e.g.*, relating to the sugar concentration during the fermentation). They may also concern the purification of product (*e.g.*, by ion-exchange chromatography) or the intrinsic performance properties of the microorganism itself.

To improve the performance properties of amino acid-producing microorganisms, methods of mutagenesis, selection and mutant selection are often employed. These methods may be used to obtain strains that are resistant to antimetabolites, such as, for example, the lysine analogue S-(2-aminoethyl)-cysteine, or which are auxotrophic for amino acids which are important in terms of regulation, and produce L-amino acids. In addition, methods of recombinant DNA technology have been used to improve the L-amino-acid-producing strains of Corynebacterium glutamicum by amplifying individual genes of amino acid biosynthesis. General articles on this subject include Kinoshita ("Glutamic Acid Bacteria," in: Biology of Industrial Microorganisms, Demain and Solomon (eds.), Benjamin Cummings, London, UK, 1985, 115-142; Hilliger, *BioTec 2*:40-44 (1991); Eggeling, *Amino Acids 6*:261-272 (1994); Jetten, *et al.*, *Crit. Rev. Biotech.15*:73-103 (1995); and Sahm, *et al.*, *Ann. New York Acad. Sci. 782*:25-39 (1996)).

Summary of the Invention

The present invention is based upon the isolation of a bacterial gene coding for the enolase enzyme and the discovery that amino acid synthesis in coryneform bacteria is increased when the activity of this enzyme is enhanced. Bacteria may be treated in a number of different ways to enhance enolase activity but, most typically enhancement will result from bacteria being transformed with an expression vector encoding the enzyme. "Enhanced" refers to an increase in enolase activity in the treated bacteria relative to the amount of activity in untreated bacteria.

In its first aspect, the present invention is directed to an isolated polynucleotide from coryneform bacteria containing a polynucleotide sequence coding for the eno gene. The polynucleotide is selected from the group consisting of: a) a polynucleotide that is at least 70% identical with a polynucleotide that codes for a polypeptide containing the amino-acid sequence of SEQ ID NO:2; and b) a polynucleotide that codes for a polypeptide containing an amino acid sequence that is at least 70 % identical (and preferably 80 or 90% identical) with the amino-acid sequence of SEQ ID NO:2. In either case, the polypeptide must exhibit the activity of the enolase enzyme and, prefereably, it is a recombinant DNA replicative in coryneform bacteria.

The term "isolated" means separated out of its natural environment. Thus, a protein or polypeptide that has been purified would be isolated for the purposes of the present invention. "Polynucleotide" in refers to polyribonucleotides and, preferably, polydeoxyribonucleotides, it being possible for these to be non-modified or modified RNA or DNA. "Polypeptides" is understood as meaning peptides or proteins which comprise two or more amino acids bonded via peptide bonds.

A preferred polynucleotide coding for the enolase enzyme is that having the nucleotide sequence of SEQ ID NO:1. The invention includes not only polynucleotides having this specific sequence but also those that are at least 70% homologous (and preferably 80 or 90% homologous) to SEQ ID NO:1 and and in which one or more of the codons are replaced with a degenerate codon or in which there is a neutral sense mutation that does not alter the activity of the enolase enzyme. In addition, the invention encompasses a corynebacteria containing the any of the polynucleotides that are a part of the present invention.

Polynucleotide sequences derived from SEQ ID NO:1 may be used as hybridization probes for RNA, cDNA and DNA, in order to isolate the full length cDNA which coding for enolase and to isolate genes ,which have a high degree of homology with the enolase gene. Such oligonucleotides serving as probes or primers (e.g. for PCR) should be at least 15, and preferably at least 30 to 50 base pairs in length.

The inventors have found that improved activity occurs when the L-glutamic acid at position 223 of SEQ ID NO:2 is replaced by another proteinogenic amino acid. Thus, the invention includes enolase proteins with include this mutation and polypeptides which code for the mutated polypeptides. In a preferred embodiment, the L-glutamic acid at positon 223 is replaced with L-lysine as shown in SEQ ID NO:4. One way to code for a polypeptide to code for this is shown herein as SEQ ID NO:3 which is similar to SEQ ID NO:1 but with an adenine at position 817.

In another aspect, the invention is directed to a method for the production an L-amino acid, by: a) fermenting a coryneform bacteria that produces the amino acid and in which the activity of the enolase enzyme is enhanced; and b) isolating the L-amino acid from either the bacteria or from the medium used to grow the bacteria. Preferably, the amino acid being produced by the method is L-lysine and the enhancement of enolase activity results from the overexpression of the eno gene. The term "overexpression" as used in this instance refers to an increase the amount of mRNA transcribed relative to the amount of transcription occurring in untreated bacteria. The coryneform bacteria may be transformed with a nucleotide sequence coding for the enolase enzyme of SEQ ID NO:2 or in which the L-glutamic acid at position 223 of SEQ ID NO:2 is replaced by another proteinogenic L-amino acid, preferably L-lysine.

In addition to exhibiting enhanced enolase activity, the bacteria used to produce amino acids may have undergone additional genetic alterations to increase production. For example, the bacteria may be engineered to over-express one more genes encoding enzymes used in the biosynthetic pathway of of said L-amino acid or metabolic paths that reduce the formation of of the amino acid, e.g. L-lysine, may be at least partially eliminated. Specific genes that may be overexpressed to increase amino acid synthesis include: dapA gene coding for dihydrodipicolinate synthase; the lysC gene coding for a feedback-resistant aspartate kinase; the gap gene coding for glyceraldehyde-3-phosphate dehydrogenase; the tpi gene

coding for triosephosphate isomerase; the pgk gene coding for 3-phosphoglycerate kinase; and the pyc gene coding for pyruvate carboxylase.

Brief Description of the Figures

Figure 1: Figure 1 is a map of plasmid pEC-XT99A.

Figure 2: Figure 2 is a map of plasmid pXT-enoex.

Detailed Description of the Invention

The present invention is based upon the development of improved methods for the production of L-amino acids, especially L-lysine, by fermentation. Unless otherwise indicated, any mention of L-lysine or lysine herein is to be understood as meaning not only the base but also salt forms of the amino acid, such as, for example, lysine monohydrochloride or lysine sulfate. This also applies with respect to other amino acids.

The invention provides a process for the production of L-amino acids, especially L-lysine, by fermentation using coryneform bacteria which, especially, already produce the desired amino acid and in which the activity of the enzyme malate:quinone oxidoreductase (mqo) is enhanced, especially by over-expression of its gene. The term "enhanced" or "enhancement" in this connection describes a change which leads to an increase in the intracellular activity of the enzyme relative to the activity seen in the unaltered microorganism. For example, enhancement may be accomplished by increasing the copy number of the gene, using a strong promoter, or using a gene or allele that codes for a corresponding enzyme having a high degree of activity, and optionally combining those measures. "Amplification" refers to a specific procedure for achieving an enhancement whereby the number of DNA molecules carrying a gene or genes, an allele or alleles, a regulatory signal or signals or any other genetic feature is increased.

The microorganisms provided by the present invention can produce L-amino acids, especially L-lysine, from glucose, saccharose, lactose, fructose, maltose, molasses, starch, cellulose or from glycerol and ethanol. They are representatives of coryneform bacteria, especially of the genus Corynebacterium. In this genus, a preferred species is Corynebacterium glutamicum. Examples of suitable strains of bacteria are as follows:

Corynebacterium	glutamicum		ATCC13032
Corynebacterium	acetoglutamicum		ATCC15806
Corynebacterium	acetoacidophilum		ATCC13870
Corynebacterium	thermoaminogenes	FERM	BP-1539
Brevibacterium	flavum		ATCC14067
Brevibacterium	lactofermentum		ATCC13869
Brevibacterium	divaricatum		ATCC14020.

Examples of suitable L-amino-acid-producing, especially L-lysine-producing, mutants and strains produced therefrom, include:

Corynebacterium	glutamicum	FERM-P	1709
Brevibacterium	flavum	FERM-P	1708
Brevibacterium	lactofermentum	FERM-P	1712
Corynebacterium	glutamicum	FERM-P	6463
Corynebacterium	glutamicum	FERM-P	6464
Corynebacterium	glutamicum	DSM	5715

Gene isolation may be accomplished by constructing a gene library of this microorganism in E. coli. Procedures for making such libraries are well known in the art (see, *e.g.*, Winnacker: Gene und Klone, Eine Einführung in die Gentechnologie, Verlag Chemie, Weinheim, Germany, 1990; Sambrook *et al.*, Molecular Cloning, A Laboratory Manual, Cold Spring Harbor Laboratory Press, 1989); Kohara, *et al.*, *Cell 50*:495-508 (1987)). Bathe *et al.* (*Mol. Gen. Genet. 252*:255-265 (1996)) describe a gene library of C. glutamicum ATCC13032 which was constructed with the aid of the cosmid vector SuperCos I (Wahl *et al.*, *Proc. Nat'l Acad. Sci. USA 84*:2160-2164 (1987)) in the E.coli K-12 strain NM554 (Raleigh *et al.*, *Nuc. Acids Res. 16*:1563-1575 (1988)). A gene library of C. glutamicum ATCC13032 was also made by Börmann *et al.* (*Mol. Microbiol. 6(3)*:317-326)) using the cosmid pHC79 (Hohn *et al.*, *Gene 11*:291-298 (1980)). A gene library of C. glutamicum may also be prepared in E. coli using plasmids such as pBR322 (Bolivar, *Life Sci. 25*:807-818 (1979)) or pUC9 (Viera *et al.*, *Gene 19*:259-268 (1982)). Suitable hosts are, in particular, those E. coli strains that are restriction- and recombination-defective, *e.g.*, strain DH5αmcr (Grant *et al.*, *Proc. Nat'l Acad. Sci. USA 87*:4645-4649 (1990)). DNA fragments can be subcloned and sequenced in vectors that are suitable for sequencing, such as those described

by Sanger *et al.* (*Proc. Natl Acad. Sci. USA 74*:5463-5467, (1977)). Using these methods, the inventors isolated the eno gene of C. glutamicum. This codes for the enzyme enolase (EC 4.2.1.11) and was determined to have the sequence shown herein as SEQ ID NO:1. The amino acid sequence of the corresponding protein was derived from the DNA sequence and is shown herein as SEQ ID NO:2.

Coding DNA sequences which correspond to SEQ ID NO:1 by the degeneracy of the genetic code are part of the invention. Conservative amino acid exchanges, such as the exchange of glycine for alanine or aspartic acid for glutamic acid, are known among experts as "sense mutations" and do not usually lead to a substantial change in the activity of the protein, *i.e.*, they are of neutral function. Similarly, it is known that changes in the N and/or C terminus of proteins typically do not substantially impair, and can even stabilize, their function (see, *e.g.*, Ben-Bassat, *et al.*, *J. Bacteriol. 169*:751-757 (1987); O'Regan, *et al.*, *Gene 77*:237-251 (1989); Sahin-Toth, *et al.*, *Prot. Sci. 3*:240-247 (1994); and Hochuli *et al.*, *Bio/Tech. 6*:1321-1325 (1988)). Amino acid sequences which are differ from SEQ ID NO:2 only with respect to differences of this type which do not substantially affect enolase activity are also part of the invention.

DNA sequences which hybridize with SEQ ID NO:1 or parts of SEQ ID NO:1 are a included within the scope of the invention, as are DNA sequences which are prepared by the polymerase chain reaction (PCR) using primers derived from SEQ ID NO:1. Such oligonucleotides typically have a length of at least 15 base pairs. Instructions for identifying DNA sequences by means of hybridization can be found, *inter alia*, in the handbook "The DIG System Users Guide for Filter Hybridization" (Boehringer Mannheim GmbH, Mannheim, Germany, (1993)) and in Liebl *et al.* (Int. J. Sys. Bacteriol. 41: 255-260 (1991)). A description of methods for amplifying DNA sequences using the polymerase chain reaction (PCR) can be found, *inter alia*, in a handbook by Gait (Oligonukleotide synthesis: a practical approach, IRL Press, Oxford, UK (1984)) and by Newton *et al.* (PCR, Spektrum Akademischer Verlag, Heidelberg, Germany (1994)).

The inventors have found that coryneform bacteria produce amino acids, in particular L-lysine, in an improved manner after over-expression of the eno gene. In order to achieve over-expression, the copy number of the eno gene can be increased, or the promoter and regulation region, which is located in front of the structural gene, can be mutated. Expression

cassettes, which are inserted in front of the structural gene, have the same effect. By means of inducible promoters it is additionally possible to increase the expression in the course of the production of L-lysine by fermentation. Expression is likewise improved by measures for lengthening the life of the m-RNA, *e.g.*, by inhibiting the rate at which enzyme is degraded. The genes or gene constructs can either be present in plasmids with different copy numbers or be integrated and amplified in the chromosome.

Alternatively, over-expression of the eno gene can be achieved by changing the composition of the bacterial growth medium and the manner in which culturing is carried out. The person skilled in the art will find a detailed description of procedures that can be followed for carrying out these objectives in a number of publications, including: Martin *et al.*, *Bio/Technology 5*:137-146 (1987); Guerrero, *et al.*, *Gene 138*:35-41 (1994); Tsuchiya, *et al.*, *Bio/Technology 6*:428-430 (1988); Eikmanns, *et al.*, *Gene 102*:93-98 (1991); EP-B 0 472 869; US 4,601,893; Schwarzer, *et al.*, *Bio/Technology 9*:84-87 (1991); Reinscheid, *et al.*, *Appl. Environment. Microbiol. 60*:126-132 (1994); LaBarre, *et al.*, *J. Bacteriol. 175*:1001-1007 (1993); WO 96/15246; Malumbres, *et al.*, *Gene 134*:15-24 (1993); Jensen *et al.*, *Biotech. Bioeng. 58*:191-195 (1998); Makrides, *Microbiol. Rev. 60*:512-538 (1996) and in other standard textbooks of genetics and molecular biology.

By way of example, the eno gene according to the invention was over-expressed with the aid of plasmids. Suitable plasmids are those which are replicated in coryneform bacteria. Suitable plasmid vectors such as pZ1 (Menkel *et al.*, *App. Envir. Microbiol. 64*:549-554 (1989)), pEKEx1 (Eikmanns *et al.*, *Gene 102*:93-98 (1991)) and pHS2-1 (Sonnen *et al.*, *Gene 107*:69-74 (1991)) are based on the the cryptic plasmids pHM1519, pBL1 and pGA1. Other plasmid vectors such as those based on pCG4 (U.S. 4,489,160), pNG2 (Serwold-Davis, *et al.*, *FEMS Microbiol. Lett. 66*:119 – 124 (1990)) and pAG1 (U.S. 5,158,891) can also be used.

In addition, it was found that the replacement of the amino acid L-glutamic acid at position 223 of the enolase enzyme (see SEQ ID NO:2) by any other proteinogenic amino acid, especially L-lysine (see SEQ ID No. 4), results in an enhancement of amino acid synthesis. Thus, coryneform bacteria that carry an enolase enzyme having such an amino acid replacement produce amino acids, especially L-lysine, in an improved manner. In a preferred embodiment, the replacement of L-glutamic acid by L-lysine is accomplished by the

substitution of guanine at position 817 of the eno gene by adenine, as shown in the nucleotide sequence of SEQ ID NO:3.

Mutation of the eno gene can be carried out using classic methods and employing mutagenic substances such as, *e.g.*, N-methyl-N'-nitro-N-nitrosoquanidine or ultraviolet light. Mutagenesis can also be performed using *in vitro* methods such as those involving treatment with hydroxyl amine (Konickova-Radochova, *Folia Microbiol. 13(3)*:226-230, Prague (1968)), mutagenic oligonucleotides (Brown, Gentechnologie für Einsteiger, Spektrum Akademischer Verlag, Heidelberg, (1993)) and the polymerase chain reaction (PCR) (Newton, *et al.*, PCR, Spektrum Adademischer Verlag, Heidelberg (1994)).

After sequencing, the eno alleles or genes may be introduced into a suitable host. Replicating plasmid vectors can be used for this. It is likewise possible to insert the eno allele or gene into the chromosome of a suitable host by gene replacement as described, for example, by Schwarzer and Pühler (*Bio/Tech. 9*:84–87 (1991)). The allele or gene in question is cloned into a vector that is not replicative for C. glutamicum and this vector is subsequently transferred via transformation or conjugation into the desired host. Insertion takes place by homologous recombination involving a first crossover event bringing about integration and a second crossover event in the target sequence bringing about excision. This method was used by Peters-Wendisch *et al.* (*Microbiol. 144*:915–927 (1998)) to replace the pyc gene of C. glutamicum with a pyc allele carrying a deletion.

In addition, the method of chromosomal gene amplification can be used (see, *e.g.*, Reinscheid *et al.*, *App. Environ. Microbiol. 60*:126–132 (1994)). In this method, the complete gene or allele is cloned into a plasmid vector that can replicate in a host (typically E. coli) but not in C. glutamicum. Potential vectors include: pSUP301 (Simon *et al.*, *Bio/Tech. 1*:784–791 (1983)); pK18mob or pK19mob (Schäfer *et al.*, *Gene 145*:69–73 (1994)); pGEM-T (Promega Corp., Madison, WI, USA); pCR2.1-TOPO (Schuman, *J. Biol. Chem. 269*:32678-84 (1994); U.S. 5,487,993); pCR®Blunt (Invitrogen Co., Gronigen, The Netherlands; Bernard *et al.*, *J. Mol. Biol. 234*:534–541 (1993)); pEM1 (Schrumpf *et al.*, *J. Bacteriol. 173*:4510–4516 (1991)); and pBGS8 (Spratt *et al.*, *Gene 41*:337–342 (1986)). The plasmid vector containing the gene or allele to be amplified is subsequently transferred by conjugation or transformation into the desired strain of C. glutamicum. Methods for carrying out

conjugation and transformation have been described by, *inter alia*: Schäfer *et al.* (*App. Environ. Microbiol. 60*:756–759 (1994)); Thierbach *et al.* (*App. Microbiol. Biotech. 29*:356–362 (1988)); Dunican, *et al.*, (*Bio/Tech. 7*:1067–1070 (1989)); and Tauch, *et al.* (*FEMS Microbiol. Lett. 123*:343–347 (1994)). After homologous recombination by means of a crossover event, the resulting strain contains at least two copies of the particular gene or allele.

Accordingly, further subject matter of the invention is constituted by coryneform bacteria that contain the enolase enzyme proteins in which the amino acid sequence shown under SEQ ID NO:2 at position 223 is replaced by another amino acid with the exception of L-glutamic acid. A further aspect of this invention is constituted by coryneform bacteria that contain a corresponding enzyme protein in which the amino acid L-glutamic acid at position 223 of the enzyme protein (see, SEQ ID NO:2) is replaced by L-lysine (see, SEQ ID N:4).

In addition to the eno gene, it can be advantageous for the production of amino acids, especially L-lysine, to over-express one or more enzymes involved in their biosynthetic pathway, in glycolysis, in the anaplerotic metabolic pathway, in the citric-acid cycle or in amino acid export. Thus, for the production of L-lysine it may be advantageous to over-express both eno and one or more of the following:

- the dapA gene coding for dihydrodipicolinate synthase (EP-B 0 197 335);
- the lysC gene coding for a feedback-resistant aspartokinase (EP-B 0387527, U.S. 5,688,671);
- the gap gene coding for glyceraldehyde-3-phosphate dehydrogenase (Eikmanns, *J. Bacteriol. 174*:6076-6086 (1992));
- the tpi gene coding for triosephosphate isomerase (Eikmanns, *J. Bacteriol. 174*:6076–6086 (1992));
- the pgk gene coding for 3-phosphoglycerate kinase (Eikmanns, *J. Bacteriol. 174*:6076–6086 (1992));
- the pyc gene coding for pyruvate carboxylase (Eikmanns, *J. Bacteriol. 174*:6076–6086 (1992)); and
- the lysE gene coding for the lysine export protein (DE-A-195 48 222).

In addition to over-expressing the eno gene, it may also be advantageous for the production of amino acids, in particular L-lysine, to eliminate undesirable side reactions (Nakayama: "Breeding of Amino Acid Producing Micro-organisms", in: Overproduction of Microbial Products, Krumphanzl, Sikyta, Vanek (eds.), Academic Press, London, UK, 1982)).

The microorganisms produced according to the invention may be cultivated continuously or discontinuously in a batch process, in a fed batch, or by a repeated fed batch process for the purpose of producing L-amino acids. A summary of cultivation methods is described in the textbook by Chmiel (Bioprozesstechnik 1. Einführung in die Bioverfahrenstechnik, Gustav Fischer, Verlag, Stuttgart, (1991)) or in the textbook by Storhas (Bioreaktoren und periphere Einrichtungen, Vieweg Verlag, Braunschweig/-Wiesbaden (1994)). The culture medium to be used must meet the requirements of the strain being used for production. Descriptions of culture media for various microorganisms are contained in the handbook Manual of Methods for General Bacteriology of the American Society for Bacteriology, Washington D.C., USA, (1981).

Examples of compounds that can be used as a carbon source include: sugars and carbohydrates such as glucose, saccharose, lactose, fructose, maltose, molasses; starch and cellulose; oils and fats such as soybean oil, sunflower oil, groundnut oil and coconut fat; fatty acids, such as palmitic acid, stearic acid and linoleic acid; alcohols such as glycerol and ethanol; and organic acids such as acetic acid. These substances may be used individually or in the form of a mixture. Examples of compounds that can be used as a nitrogen source include: organic nitrogen-containing compounds such as peptones, yeast extract, meat extract, malt extract, corn steep liquor, soybean flour and urea; or inorganic compounds such as ammonium sulfate, ammonium chloride, ammonium phosphate, ammonium carbonate and ammonium nitrate. The nitrogen sources may be used individually or in the form of a mixture.

Compounds that can be use as a phosphorus source include potassium dihydrogen phosphate and dipotassium hydrogen phosphate or the corresponding sodium-containing salts. The culture medium must also contain salts of metals, such as, for example, magnesium sulfate or iron sulfate, which are necessary for growth. Finally, essential growth substances such as amino acids and vitamins may be used in addition to the above-mentioned

substances. Moreover, suitable pre-stages may be added to the culture medium. The mentioned substances may be added to the culture in the form of a single batch or may be fed in a suitable manner during the cultivation.

In order to control the pH of the culture, basic compounds, such as sodium hydroxide, potassium hydroxide, ammonia, or acid compounds, such as phosphoric acid or sulfuric acid, can be used. For controlling the development of foam, antifoams, such as, for example, fatty acid polyglycol esters, may be added.

Plasmid stability can be maintained by adding substances having a selective action, for example antibiotics, to the medium. In order to maintain aerobic conditions, oxygen or oxygen-containing gas mixtures, such as, for example, air, are introduced into the culture.

The temperature of the culture is normally from 20°C to 45°C and preferably from 25°C to 40°C. Culturing is continued until a maximum of the desired L-amino acid has formed. That aim is normally achieved within a period of from 10 hours to 160 hours. Analysis of L-amino acids may be carried out by anion- exchange chromatography with subsequent ninhydrin derivatization, as described by Spackman *et al.* (*Analyt. Chem. 30*:1190 (1958)).

The invention may be further understood by reference to the following non-limiting examples.

Examples

Example 1: Production of a Genomic Cosmid Gene Bank from Corynebacterium Glutamicum ATCC 13032

Chromosomal DNA from Corynebacterium glutamicum ATCC 13032 was isolated as described in Tauch *et al.*, (*Plasmid 33*:168–179 (1995)) and partially cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, product description Sau3AI, code No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, product description SAP, code No. 1758250). The DNA of the cosmid vector SuperCos1 (Wahl *et al.* *Proc. Nat'l Acad. Sci. USA 84*:2160–2164 (1987)) obtained from the Stratagene company (La

Jolla, USA, product description SuperCos1 cosmid Vektor Kit, Code No. 251301) was cleaved with the restriction enzyme XbaI (Amersham Pharmacia, Freiburg, Germany, product description XbaI, code No. 27-0948-02) and also dephosphorylated with shrimp alkaline phosphatase. The cosmid DNA was subsequently cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, product description BamHI, code No. 27-0868-04). The cosmid DNA treated in this manner was mixed with the treated ATCC13032 DNA and the batch treated with T4 DNA ligase (Amersham Pharmacia, Freiburg, Germany, product description T4 DNA ligase, code No. 27-0870-04).

The ligation mixture was subsequently packed in phages with the aid of Gigapack II XL packing extracts (Stratagene, La Jolla, USA, product description Gigapack II XL packing extract code No. 200217). For the infection of the E. coli strain NM554 (Raleigh *et al., Nucl. Ac. Res. 16:*1563-1575 (1988)), the cells were taken up in 10 mM $MgSO_4$ and mixed with an aliquot of the phage suspension. The infection and titering of the cosmid library were carried out as described by Sambrook, *et al.* (Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989), the cells being plated out on LB agar (Lennox, *Virology 1*:190 (1955)) with 100 µg/ml ampicillin. After incubation overnight at 37°C, recombinant individual clones were selected.

Example 2: Isolation and Sequencing of the Eno Gene

The cosmid DNA of an individual colony was isolated with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) in accordance with the manufacturer's instructions and partly cleaved with the restriction enzyme Sau3AI (Amersham Pharmacia, Freiburg, Germany, Product Description Sau3AI, Product No. 27-0913-02). The DNA fragments were dephosphorylated with shrimp alkaline phosphatase (Roche Molecular Biochemicals, Mannheim, Germany, Product Description SAP, Product No. 1758250). After separation by gel electrophoresis, cosmid fragments in the size range of 1500 to 2000 bp were isolated with the QiaExII Gel Extraction Kit (Product No. 20021, Qiagen, Hilden, Germany). The DNA of the sequencing vector pZero-1, obtained from the company Invitrogen (Groningen, The Netherlands, Product Description Zero Background Cloning Kit, Product No. K2500-01) was cleaved with the restriction enzyme BamHI (Amersham Pharmacia, Freiburg, Germany, Product Description BamHI, Product No. 27-0868-04). The ligation of the cosmid fragments in the sequencing vector pZero-1 was carried out as described by Sambrook, *et al.* (Molecular Cloning: A laboratory Manual, Cold Spring

Harbor, 1989), the DNA mixture being incubated overnight with T4 ligase (Pharmacia Biotech, Freiburg, Germany). This ligation mixture was subsequently electroporated into the E. coli strain DH5αMCR (Grant, *Proc. Natl. Acad. Sci. USA 87*:4645-4649 (1990); Tauch, *et al.*, *FEMS Microbiol. Lett. 123*:343-7 (1994)) and plated out onto LB agar (Lennox, *Virology 1*:190 (1955)) with 50 µg/ml zeocine. The plasmid preparation of the recombinant clones took place using the Biorobot 9600 (Product No. 900200, Qiagen, Hilden, Germany). Sequencing took place according to the dideoxy chain termination method of Sanger, *et al.* (*Proc. Natl. Acad. Sci. U.S.A. 74*:5463-5467 (1977)) with modifications according to Zimmermann *et al.* (*Nucl. Ac. Res. 18*:1067 (1990)). The "RR dRhodamin Terminator Cycle Sequencing Kit" of PE Applied Biosystems (product No. 403044, Weiterstadt, Germany) was used. The gel electrophoretic separation and analysis of the sequencing reaction took place in a "Rotiphorese NF Acrylamid/Bisacrylamid" gel (29:1) (product No. A124.1, Roth, Karlsruhe, Germany) with the "ABI Prism 377" sequencing device of PE Applied Biosystems (Weiterstadt, Germany).

The raw sequence data obtained was subsequently processed using the Staden program packet (*Nucl. Ac. Res. 14*:217– 231 (1986)) version 97-0. The individual sequences of the pZerol derivatives were assembled to a cohesive contig. The computer-supported coding range analysis was prepared with the program XNIP (Staden, *Nucl. Ac. Res. 14*:217–231 (1986)). Further analyses were carried out with the "BLAST search programs" (Altschul *et al.*, *Nucl. Ac. Res. 25*:3389–3402 (1997)) against the non-redundant databank of the "National Center for Biotechnology Information" (NCBI, Bethesda, MD, USA).

The nucleotide sequence obtained is shown as SEQ ID NO:1. An analysis of the nucleotide sequence yielded an open reading frame of 1275 base pairs that was designated as the "eno gene." The gene codes for a protein of 425 amino acids.

Example 3: Production of Shuttle Vector pXT-enoex for Enhancing the Eno Gene in C. Glutamicum

3.1. Cloning of the eno gene

Chromosomal DNA was isolated from the strain ATCC 13032 according to the method of Eikmanns *et al.* (*Microbiology 140*:1817–1828 (1994)). Based on the sequence of

the eno gene known from Example 2, the following oligonucleotides were selected for the polymerase chain reaction:

eno-ex1 (SEQ ID NO:5):

5' TTG GCA TAG GAG GCC ACA GT 3'

eno-ex2 (SEQ ID NO:6):

5' ATT TAG CCC TGA AAG CGT GG 3'.

The primers shown were synthesized by the ARK Scientific GmbH Biosystems company (Darmstadt, Germany) and the PCR reaction was carried out according to the standard PCR method of Innis, *et al.* (PCR Protocols. A Guide to Methods and Applications, Academic Press, 1990) with Pwo polymerase of the Roche Diagnostics GmbH company (Mannheim, Germany). The primers make possible, with the aid of the polymerase chain reaction, the amplification of a DNA fragment approximately 1.3 kb large that carries the eno gene. The DNA sequence of the amplified DNA fragment was tested by sequencing.

3.2. Production of the E. coli – C. glutamicum shuttle vector pEC-XT99A

The E. coli expression vector pTRAC99A (Amman *et al.*, *Gene 69*:301 315 (1988)) was used as starting vector for the construction of the E. coli - C. glutamicum shuttle expression vector pEC-ST99A. After BspHI restriction cleavage (Diagnostics GmbH, Mannheim, Germany, product description BspHI, product No. 1467123) and subsequent Klenow treatment (Amersham Pharmacia Biotech, Freiburg, Germany, product description Klenow Fragment of DNA Polymerase I, product No. 27-0928-01; method according to Sambrook, *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989), the ampicillin resistance gene (bla) was replaced by the tetracycline resistance gene of the C. glutamicum plasmid pAG1 (GenBank Accession No. AF121000). To this end the resistance-gene-carrying construct was cloned as an AluI fragment (Amersham Pharmacia Biotech, Freiburg, Germany, product description AluI, product No. 27-0884-01) into the linearized E. coli expression vector pTRC99A. The ligation was carried out as described by Sambrook et al., 1989, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor) and the DNA mixture incubated overnight with T4 ligase (Amersham Pharmacia Biotech, Freiburg, Germany, product description T4-DNA ligase, product No. 27-0870-04). This ligation mixture was subsequently electroporated into the E. coli strain DH5αmcr (Grant, *Proc. Natl.*

Acad. Sci. USA 87:4645–4649 (1990); Tauch, *et al.*, *FEMS Microbiol. Lett. 123*:343-7 (1994)). The constructed E. coli expression vector was designated with pXT99A.

The plasmid pGA1 (Sonnen *et al.*, *Gene, 107*:69–74 (1991)) was used as the basis for cloning a minimal replicon from Corynebacterium glutamicum. A fragment 3484 bp in size was cloned into the vector pK18mob2 (Tauch *et al.*, *Arch. Microbiol. 169*:303–312 (1998)) fragmented with SmaI and PstI (Amersham Pharmacia Biotech, Freiburg, Germany, product description SmaI, product No. 27-0942-02) by means of BalI/PstI restriction cleavage (Promega GmbH, Mannheim, Germany, product description BalI, product No. R6691; Amersham Pharmacia Biotech, Freiburg, Germany, product description, PstI, product No. 27-0976-01) of vector pGA1. A fragment 839 bp in size was deleted by means of BamHI/XhoI restriction cleavage (Amersham Pharmacia Biotech, Freiburg, Germany, product description BamHI, product No. 27-086803, product description XhoI, product No. 27-0950-01) and subsequent Klenow treatment (Amersham Pharmacia Biotech, Freiburg, Germany, product description Klenow fragment of DNA polymerase I, product No. 27-0928-01; method according to Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989). The C. glutamicum minimal replicon was cloned as 2645 bp fragment into the E. coli expression vector pXT99A from the construct religated with T4 ligase (Amersham Pharmacia Biotech, Freiburg, Germany, product description T4-DNA ligase, product No. 27-0870-04). To this end the DNA of the construct carrying the minimal replicon was cleaved with the restriction enzymes KpnI (Amersham Pharmacia Biotech, Freiburg, Germany, product description KpnI, product No. 27-0908-01) and PstI (Amersham Pharmacia Biotech, Freiburg, Germany, product description PstI, product No. 27-0886-03) and a 3'-5'-exonuclease treatment (Sambrook *et al.*, Molecular Cloning: A Laboratory Manual, Cold Spring Harbor, 1989) was subsequently carried out by means of Klenow polymerase (Amersham Pharmacia Biotech, Freiburg, Germany, product description Klenow fragment of DNA polymerase I, product No. 27-0928-01).

In a parallel batch, the E. coli expression vector pXT99A was cleaved with the restriction enzyme RsrII (Roche Diagnostics, Mannheim, Germany, product description RsrII, product No. 1292587) and prepared for ligation with Klenow polymerase (Amersham Pharmacia Biotech, Freiburg, Germany, Klenow fragment of DNA polymerase I, product No. 27-0928-01). The ligation of the minimal replicon with vector construct pXT99A was carried out as described by Sambrook *et al.*, (Molecular Cloning: A Laboratory Manual, Cold Spring

Harbor, 1989) during which the DNA mixture was incubated overnight with T4 ligase (Amersham Pharmacia Biotech, Freiburg, Germany, product description T4-DNA ligase, product No. 27-0870-04).

The E. coli C. glutamicum shuttle expression vector pEC-XT99A constructed as described above was transferred by electroporation (Liebl, *et al.*, *FEMS Microbiol. Lett. 53*:299–303 (1989)) into C. glutamicum DSM5715. The selection of the transformants took place on LBHIS agar consisting of 18.5 g/l brain-heart infusion bullion, 0.5 M sorbitol, 5 g/l bacto-trypton, 2.5 g/l bacto-yeast extract, 5 g/l NaCl and 18 g/l bacto agar that had been supplemented with 5 mg/l tetracycline. The incubation was carried out 2 days at 33°C.

Plasmid DNA was isolated from a transformant according to the customary methods (Peters-Wendisch, *et al.*, *Microbiol. 144*:915–926 (1998)), cut with restriction endonuclease HindIII and the plasmid checked by subsequent agarose gel electrophoresis. The plasmid construct obtained in this manner was designated pEC-XT99A and is shown in figure 1. The strain obtained by electroporation of plasmid pEC-XT99A into Corynebacterium glutamicum strain DSM5715 was named DSM5715/pEC-XT99A and deposited as DSM 12967 in the German Collection for Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) according to the Treaty of Budapest.

3.3. Cloning of eno in the E. coli – C. glutamicum shuttle vector pEC-XT99A

The E. coli – C. glutamicum shuttle vector pEC-XT99a described in example 3.2 was used as vector. DNA of this plasmid was completely cleaved with restriction enzyme Ecl136II and subsequently dephosphorylated with shrimp alkaline phosphatase (Roche Diagnostics GmbH, Mannheim, Germany, product description SAP, product No. 1758250).

The eno fragment obtained as described in example 3.1 was mixed with the prepared vector pEC-XT99A and the batch treated with T4-DNA ligase (Amersham Pharmacia Biotech, Freiburg, Germany, product description T4-DNA ligase, Code No. 27-0870-04). The ligation batch was transformed into the E. coli strain DH5αmcr (Grant, *Proc. Natl. Acad. Sci. USA, 87*:4645-4649 (1990)). The selection of plasmid-carrying cells took place by plating the transformation batch out onto LB agar with 5 mg/l tetracycline. After incubation overnight at 37°C, recombinant individual clones were selected. Plasmid DNA was isolated

from a transformant with the Qiaprep Spin Miniprep Kit (Product No. 27106, Qiagen, Hilden, Germany) according to the instructions of the manufacturer and cleaved with the restriction enzymes EcoRI and XbaI in order to check the plasmid by subsequent agarose gel electrophoresis. The plasmid obtained was named pXT-enoex and is shown in figure 2.

Example 4: Transformation of the Strain DSM5715 with the Plasmid pXT-enoex

The strain DSM5715 was transformed with the plasmid pXT-enox using the electroporation method described by Liebl *et al.*, (*FEMS Microbiol. Lett. 53*:299–303 (1989)). The selection of the transformants took place on LBHIS agar consisting of 18.5 g/l brain-heart infusion bullion, 0.5 M sorbitol, 5 g/l bacto-tryptone, 2.5 g/l bacto-yeast extract, 5 g/l NaCl and 18 g/l bacto-agar that had been supplemented with 5 mg/l tetracycline. The incubation took place for 2 days at 33°C.

Plasmid DNA was isolated from a transformant according to standard methods (Peters-Wendisch *et al.*, *Microbiol. 144*:915–927 (1998)), cut with the restriction endonucleases EcoRI and XbaI and the plasmid checked by subsequent agarose gel electrophoresis. The strain obtained was named DSM5715/pXT-enoex.

Example 5: Production of Lysine

The C. glutamicum strain DSM5715/pXT-enoex obtained in Example 4 was cultivated in a nutrient medium suitable for the production of lysine and the lysine content determined in the culture residue. To this end, the strain was first incubated on an agar plate with the appropriate antibiotic (brain-heart agar with tetracycline (5 mg/l)) for 24 hours at 33°C. Starting with this agar-plate culture, a preculture was inoculated (10 ml medium in a 100 ml Erlenmeyer flask). The full medium CgIII was used as medium for the preculture.

Medium Cg III	
NaCl	2.5 g/l
Bactopeptone	10 g/l
Bacto-yeast extract	10 g/l
Glucose (autoclaved separately)	2 % (w/w)

The pH was adjusted to pH 7.4.

Tetracycline ((5 mg/l) was added to this mixture. The preculture was incubated for 16 hours at 33°C at 240 rpm on an agitator. A main culture was inoculated from this preculture so that the initial OD (660 nm) of the main culture was 0.05. Medium MM was used for the main culture.

Medium MM	
CSL (corn steep liquor)	5 g/l
MOPS (morpholinopropane sulfonic acid)	20 g/l
Glucose (autoclaved separately)	100 g/l
$(NH_4)_2SO_4$	25 g/l
KH_2PO_4	0.1 g/l
$MgSO_4 * 7 H_2O$	1.0 g/l
$CaCl_2 * 2 H_2O$	10 mg/l
$FeSO_4 * 7 H_2O$	10 mg/l
$MnSO_4 * H_2O$	5.0 mg/l
Biotin (sterilized by filtration)	0.3 mg/l
Thiamine * HCl (sterilized by filtration)	0.2 mg/l
L-leucine (sterilized by filtration)	0.1 g/l
$CaCO_3$	25 g/l

CSL, MOPS and the saline solution were adjusted with ammonia water to pH 7 and autoclaved. The sterile substrate- and vitamin solutions were then added along with the $CaCo_3$, that had been autoclaved dry. The cultivation took place in 10 ml volume in a 100 ml Erlenmeyer flask with flow spoilers. Tetracycline (5 mg/l) was added. Incubation was performed at 33°C and 80 % air humidity. After 72 hours, the OD was determined at a measuring wavelength of 660 nm with a Biomek 1000 (Beckmann Instruments GmbH, Munich). The lysine amount formed was determined with an amino-acid analyzer of the Eppendorf-BioTronik company (Hamburg, Germany) by ion-exchange chromatography and postcolumn derivatization with ninhydrin detection. Table 1 shows the result of the test.

Table 1

Strain	OD (660)	Lysine – HCl g/l
DSM5715/pEC-XT99A	7.4	15.5
DSM5715/pXT-enox	7.5	16.5

Abbreviations

The abbreviations and designations used have the following meanings.

per:	Gene for monitoring the copy number from pGA1
oriV:	ColE1-like origin from pMB1
rep:	Plasmid-coded replication origin from C. glutamicum plasmid pGA1
Ptrc:	trc promoter from pTRC99A
T1, T2:	Terminator regions 1 and 2 from pTRC99A
lacIq:	repressor gene of the Lac operon
Tet:	Resistance gene for tetracycline
eno:	Enolase gene eno from C. glutamicum
EcoRI:	Cleavage site of restriction enzyme HindIII
XbaI:	Cleavage site of restriction enzyme XbaI